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Research Paper

Phytotoxic Allelochemicals From Roots and Root Exudates of Leafy Spurge (*Euphorbia esula* L.)

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phytotoxicity, allelochemicals, roots, root exudates, jatrophane diterpenes, kansuinine B, ellagic acid derivatives, leafy spurge, *Euphorbia esula*, *Arabidopsis thaliana*

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NOTE

Supplementary Material can be found at:
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ABSTRACT

Invasive plants are a widespread problem but the mechanisms used by these plants to become invasive are often unknown. The production of phytotoxic natural products by invasive weeds is one mechanism by which these species may become successful competitors. Here, we conducted a bioactivity-driven fractionation of root extracts and exudates from the invasive plant leafy spurge (*Euphorbia esula* L.), and structurally characterized jatrophane diterpenes and ellagic acid derivatives. Ellagic acid derivatives and one of the jatrophane diterpenes, esulone A, have been previously reported from leafy spurge, but another of the jatrophane diterpenes, kasuinine B, has not. We show that these compounds are phytotoxic but affect plants in different ways, either inducing overall plant necrosis or reducing root branching and elongation.

INTRODUCTION

Leafy spurge (*Euphorbia esula* L.), an exotic perennial weed, is a Eurasian native that has invaded more than 1.1 million hectares of pastureland in southern Canada and the north-central United States.[1] Leafy spurge reproduces both sexually and rhizomatously. Its seed capsules explode, sending seeds up to 5 m from the parent and its seeds can remain viable for at least 8 years.[2] After establishment, its roots can reach 4.25 m in depth.[3] Leafy spurge aboveground tissue contains white sticky latex as well as chemicals that deter grazing by cattle.[4] Soil collected from leafy spurge populations inhibited the growth of tomato (*Lycopersicon esculentum* Mil.) seedlings, suggesting that leafy spurge may produce phytotoxins that accumulate in the soil.[5] Further, addition of relatively small quantities of leafy spurge leaf and root tissue to soil (0.1 to 1.0% (w/w)) inhibited growth of both tomato and crabgrass (*Digitaria sanguinalis* (L.) Scop.) plants, suggesting that at least some phytotoxins from leafy spurge may come from decaying tissue.[5]

Studies of leafy spurge biochemistry have identified several phytotoxins, including the relatively strong phytotoxins kaempferol-3-glucuronide and 1-hexaconsanol in aqueous extracts of the whole plant,[6] and moderately phytotoxic esulone jatrophane diterpenes in leafy spurge roots.[7,8,9] However, in a comparison of ether extracts of leafy spurge roots from North Dakota, Montana, Oregon, and Austria (where the leafy spurge is not a problem weed), only the North Dakota samples had high concentrations of jatrophane diterpenes.[9] 3,3'-Di-O-methylellagic acid 4-β-D-xyloside and 3,3'-di-O-methylellagic acid 4-D-glucoside have also been isolated from leafy spurge roots, but the phytotoxicity of these compounds has not been assessed due to poor solubility.[7] Further, using bioassay-guided fractionations of aboveground tissue extracts, ingenol diterpenes have been identified as toxins in leafy spurge that may affect cattle.[10,11]

Recently, several studies have focused on the isolation and characterization of potentially allelopathic substances released by plant roots.[12] Therefore, we examined leafy spurge root exudates for allelochemicals, and we tested leafy spurge root tissue for additional phytotoxins, using bioactivity-directed fractionation and an *Arabidopsis thaliana* seedling bioassay.

MATERIALS AND METHODS

Leafy spurge root exudates. Leafy spurge (*Euphorbia esula* L.) seeds collected from populations around Fort Collins, Colorado, USA in September 2004, were surface-sterilized with 50% bleach for 30 minutes and germinated on solid Murashige and Skoog (MS) medium[13] in a 25°C incubator with a 16 h/8 h day/night schedule. Fifty seedlings were

transferred into 400 ml of liquid MS medium in a 1 L Ehrlenmeyer flask. After six weeks, the plants were treated with chitosan, a root exudate elicitor.[14] Chitosan is similar to chitin, which is a component of fungal cell walls and is likely present in most field soils. Thus, chitosan is most likely to elicit anti-fungal plant responses, but may also elicit other plant responses. Chitosan was dissolved in 0.1 N acetic acid and the solution was adjusted to pH 5.8 with 1 M NaOH. The chitosan was then applied to the MS medium to create a 0.012% chitosan solution. Three days after chitosan treatment, the MS medium containing leafy spurge root exudates was collected from the flask and extracted with chloroform and then ethyl acetate (see below). The remaining aqueous phase and the organic extracts were evaporated in a vacuum and stored frozen until the phytotoxicity assays were conducted.

Extraction and isolation of allelochemicals from leafy spurge root exudates. One liter of the leafy spurge liquid growth medium was evaporated to 750 ml and then extracted three times with 750 ml portions of chloroform. The chloroform layers were combined and evaporated to dryness in a vacuum. The aqueous phase was next extracted three times with 750 ml of ethyl acetate, and the ethyl acetate layers were combined and evaporated in a vacuum. The aqueous phase remaining was concentrated in a vacuum to leave a viscous solution. Phytotoxicity tests (see below) showed activity only in the chloroform-extracted residue (97 mg). Purification of this residue was attempted by column chromatography over RediSep silica gel flash columns on a CombiFlash Retrieve (Isco, Inc.) instrument.

Extraction and isolation of allelochemicals from leafy spurge roots. Air-dried leafy spurge roots collected from populations around Fort Collins, Colorado, USA in October 2005 were ground into a powder and 4.96 Kg were extracted with 3.2 L of methanol. The methanol extract was evaporated under vacuum, suspended in water and extracted with chloroform and ethyl acetate as described above. The chloroform extract (242 g) was divided into 15 fractions through a silica gel (40–63 μm) column (Ø5.5 x 80 cm), eluting with a $CHCl_3$-Me_2CO gradient. The fractions were tested for phytotoxic activity (see below) and active fractions were purified using a CombiFlash Retrieve (Isco, Inc.) instrument as above. The ethyl acetate extract was macerated with MeOH, filtered, and the methanol evaporated to leave a brown, gummy mass (2.02 g) which was also chromatographed on a CombiFlash Retrieve instrument.

Bioassays with *Arabidopsis* plants. Arabidopsis (*Arabidopsis thaliana*) Columbia-0 seeds obtained from Lehle Seeds (Cat. No. WT-02-36-01) were surface-sterilized with 50% bleach for 20 minutes, and germinated on solid MS medium in a 25°C incubator with a 16 h/8 h day/night schedule. Seven-day-old plants were transferred into 1 ml of liquid MS medium in 12 or 24-well plates (VWR Scientific). After 24 hours, the plants were treated with six concentrations of leafy spurge crude root exudates, the chloroform and ethyl acetate extracts of the root exudates, and the root exudate aqueous phase (0, 20, 50, 100, 200, and 500 μg ml^{-1}), with four replicates per treatment. The crude root exudates and the aqueous phase were applied directly to the liquid medium in which the plants were growing. The chloroform and ethyl acetate extracts of the root exudates were resuspended in 100% methanol, filtered with a 0.2 μm acrodisc syringe filter (VWR Scientific), and applied to fresh 12-well plates. The methanol was then allowed to evaporate to avoid effects on the plants. Once the methanol had evaporated, 1 ml of liquid MS media was added to each well and Arabidopsis plants were transferred to the wells. Extracts of dried root tissue were treated similarly, except that crude fractions from column chromatography separations and isolated pure compounds were not always methanol soluble. These were dissolved in dimethylsulfoxide (DMSO), filtered with a 0.2 μm acrodisc syringe filter and added into the wells at varying concentrations; the same amount of DMSO was used in the wells with Arabidopsis plants as a solvent control. Comparison to media-only controls indicated that the concentration of DMSO applied did not affect Arabidopsis growth (ANOVA, $F_{1,48}$=2.12, p = 0.15). Effects of tested materials on root development and plant growth were examined and documented by photography at intervals. Seven days after treatment, plants were removed from the medium, blotted dry and weighed. Relationships between concentrations of applied materials and fresh plant weight were examined with linear regression analysis. Dunnett's one-sided t-test was used for pairwise comparisons between particular treatment concentrations and the controls. Statistical analyses were conducted using SAS statistical software (version 9.1).

Instrumentation for structure determinations. Ultraviolet spectra were detected with an Agilent 8453 instrument. Infrared spectra were determined in KBr on an AVATAR 320 FT-IR instrument. Mass spectra (MS) were recorded on a Finnegan LC QduO spectrometer. Nuclear magnetic resonance (NMR) spectra (1D and 2D) were obtained on a Varian INOVA 400 instrument in deuteriochloroform or D-6 DMSO. LC-MS analysis was conducted on a Dionex system composed of P680 pump, and ASI-100 autosampler, and a UVD170U UV detector. This was coupled to a Thermo Finnigan Surveyor MSQ mass spectral detector. Separation was performed on a Dionex Acclaim 120 C18 column (5 μm, 4.6 x 150 mm) using gradient elution. Solvent A was water + 0.1% v/v acetic acid and solvent B was methanol + 0.1% acetic acid. Compounds were eluted at a 0.7 mL/min flow rate for three minutes at 10% B, a linear gradient to 90% B over 40 minutes, and held at 90% B for 8 minutes. UV detection was recorded at 254, 280 and 310 nm. Ionization for MS analysis was performed in both positive and negative ion mode using elecrospray ionization with a nitrogen flow at 80 psi, a cone voltage of 70 V, needle voltage of 3 kV, and cone temperature of 600°C. Mass data were collected over the range of the gradient program at a rate of one scan per second.

RESULTS

Identification of allelochemicals. Treatment with the leafy spurge growth medium at a concentration of 50% v/v (i.e., plants were grown in a one to one mixture of leafy spurge growth medium and fresh MS medium) slightly but significantly reduced seven-day-old Arabidopsis fresh weight (Fig. 1), suggesting that leafy spurge root exudates may contain phytotoxic compounds. Treatment with 100% v/v (i.e., plants were grown in pure leafy spurge growth medium) reduced Arabidopsis biomass by 70%. Treatment with 200 μg ml^{-1} of the chloroform-extract of the root exudates also reduced Arabidopsis biomass by 60% (Fig. 1). Treatment with the ethyl acetate extract and aqueous phase from the root exudates did not affect Arabidopsis growth or survival (data not shown). We attempted chromatographic purification of the small amounts of residues in the chloroform and ethyl acetate extracts. These purifications were unsuccessful, so instead we used leafy spurge root material to purify and identify phytotoxins that we could then look for in the root exudates.

Treatment with the chloroform extract, ethyl acetate extract, and aqueous phase of the leafy spurge root material significantly reduced seven-day-old Arabidopsis fresh weight (Fig. 2). We focused on the chloroform extract of the root material because only the chloroform

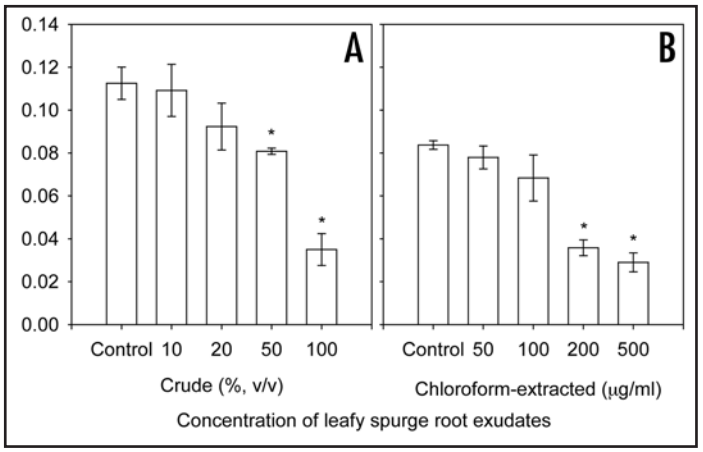

Figure 1. Phytotoxic activity of the crude (A) and chloroform-extracted (B) root exudates from leafy spurge (*Euphorbia esula L.*) on seven-day-old *Arabidopsis thaliana*. Five concentrations (0 to 100% v/v) of the crude leafy spurge root exudates in leafy spurge growing medium (A) and five concentrations (0 to 500 μg ml-1) of the chloroform extracted root exudates (B) were applied to *A. thaliana* plants growing in liquid MS medium. Both the crude exudates and the chloroform-extracted exudates significantly reduced *A. thaliana* biomass (linear regression analysis, crude root exudates, $F_{1,22}$ = 52.56, p < 0.0001; chloroform-extracted exudates, $F_{1,10}$ = 33.83, p = 0.0002). Means significantly lower than the controls are indicated with (*) (Dunnett's one-sided t-test, p < 0.05). Error bars are one standard error of the mean. N = 4.

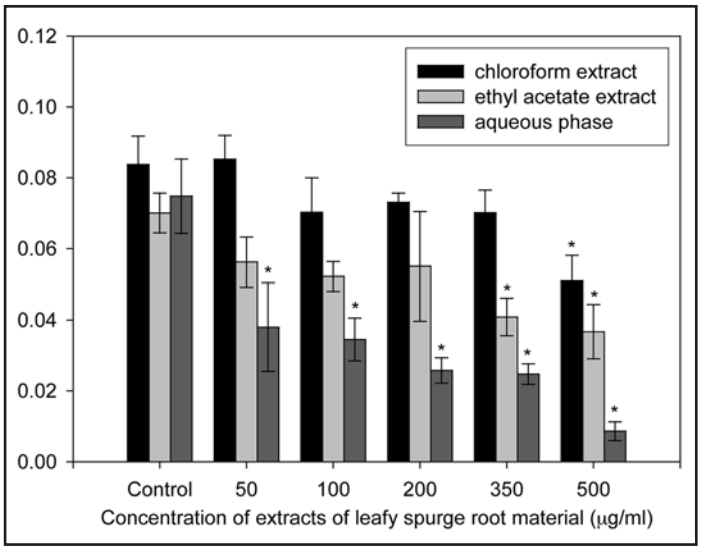

Figure 2. Phytotoxic activity of extracts from leafy spurge (*Euphorbia esula* L.) root material on seven-day-old Arabidopsis thaliana. Chloroform and ethyl acetate were used to extract organic compounds from the roots. Six concentrations (0 to 500 μg ml-1) of the chloroform and ethyl acetate extracts and the remaining aqueous phase were applied to *A. thaliana* plants growing in liquid MS medium. Effects of the extracts and remaining aqueous phase on *A. thaliana* plants were evaluated using linear regression analysis. All three extracts significantly reduced *A. thaliana* biomass (Chloroform extract: $F_{1,22}$ = 13.09, p = 0.0015. Ethyl acetate extract: $F_{1,22}$ = 9.56, p = 0.0053. Aqueous phase: $F_{1,22}$ = 21.51, p = 0.0001). Means significantly lower than the controls are indicated with (*) (Dunnett's one-sided t-test, p < 0.05). Error bars are one standard error of the mean. N = 4.

extract of the root exudates showed evidence of phytotoxins. We were mainly interested in identifying phytotoxins in the root exudates, which are more likely to be ecologically important than compounds found only in the roots. Of the 15 fractions from the chloroform extract of the root material, fractions 6-9, 11-12 and 14 were phytotoxic to *Arabidopsis* (data not shown). Comparative analyses showed that fraction 9 contained the same major compounds that were present in the chloroform extract of the root exudates, while this was less so for other fractions. Therefore, we concentrated on fraction 9 for compound purification. Fraction 9 (6.47 g) was rechromatographed on a 120 g RedSep silica gel flash column by eluting with hexane/acetone 75:25. This resulted in isolation of esulone A (1; 90 mg), kansuinine B (2; 30 mg) and 3,3',4-tri-O-methylellagic acid (3; 25 mg) (Figs. 2 and 3). Rechromatography of the 2.02 g ethyl acetate extracted residue (RediSep silica gel; chloroform/methanol 90:10) yielded ellagic acid (4; 12 mg), 4-*O*-[β-D-xylopyranosyl]-3, 3'-di-*O*-methylellagic acid (5; 90 mg), and 3,3'di-*O*-methylellagic acid (6; 33 mg) (Fig. 3). Structures of the isolates were identified by comparison of NMR and mass spectra with data from the literature (1[8], 2[15], 3[16], 4[17], 5[16], 6[16]).

LC-MS was employed using the compounds isolated from the roots to determine whether these compounds were also present in the root exudates. Kansuinine B (2; Rt 39.39 min) and the trimethylellagic acid (3; Rt 36.51 min) were detected in the chloroform extract of the root exudates. The dimethylellagic acid (6; Rt 39.7 min) and the xylopyranosyl ellagic acid derivative (5; Rt 31.2 min) were detected in the ethyl acetate extract of the root exudates. Esulone A (1) and ellagic acid (4) were not detected in either of the extracts derived from the root exudates of leafy spurge; however, this does not rule out that these compounds could be present in the root exudates in lower concentrations.

Phytotoxicity bioassays. Phytotoxic tests of the purified compounds on Arabidopsis showed that kansuinine B (2) was very active (Fig. 4 and Supp. Fig. 1). Plants were killed at concentrations ≥50 μg ml^{-1} and plant fresh weight was significantly decreased at concentrations ≥5 μg ml^{-1} (Fig. 5). Kansuinine B inhibited both roots and shoots in a concentration dependent manner.

Treatment of seedlings with 100 and 200 μg ml^{-1} of ellagic acid (4) (Supp. Fig. 2), 20, 100 and 200 μg ml^{-1} of 3,3'-di-O-methylellagic acid (6) and 200 μg ml^{-1} of 3,3',4'-tri-O-methylellagic acid (3) significantly reduced Arabidopsis fresh weight compared to DMSO controls, but not to as great a degree as treatment with kansuinine B (2) (Fig. 4). Moreover, plants treated with 200 μg ml^{-1} of compounds 3, 4, and 6 did not die, in contrast to plants treated with kansuinine B (2). After 12 hrs of treatment with the highest concentration of ellagic acid (200 μg ml^{-1}), plant roots darkened slightly and exhibited decreased branching and a 30% reduction in root elongation compared to the control plants (regression, $F_{1,18}$=6.38, p=0.0212; Supp. Fig. 2). Thus, the effect of ellagic acid on plant fresh weight appeared to be due mainly to root damage. Treatment with 200 μg ml^{-1} of the two methoxy derivatives 3 and 6 also appeared to reduce root elongation and branching but the roots did not change color (Qin B, personal observation). Esulone A (1) and the xyloside derivative 5 did not show phytotoxic activity against Arabidopsis at concentrations up to 200 μg ml^{-1} (Fig. 4).

DISCUSSION

Leafy spurge roots apparently contain at least two different types of compounds with effects on Arabidopsis seedlings: jatrophane diterpenes and ellagic acid derivatives. One of the diterpenes, kansuinine B (2), was strongly phytotoxic, killing seedlings at 200 μg ml^{-1},

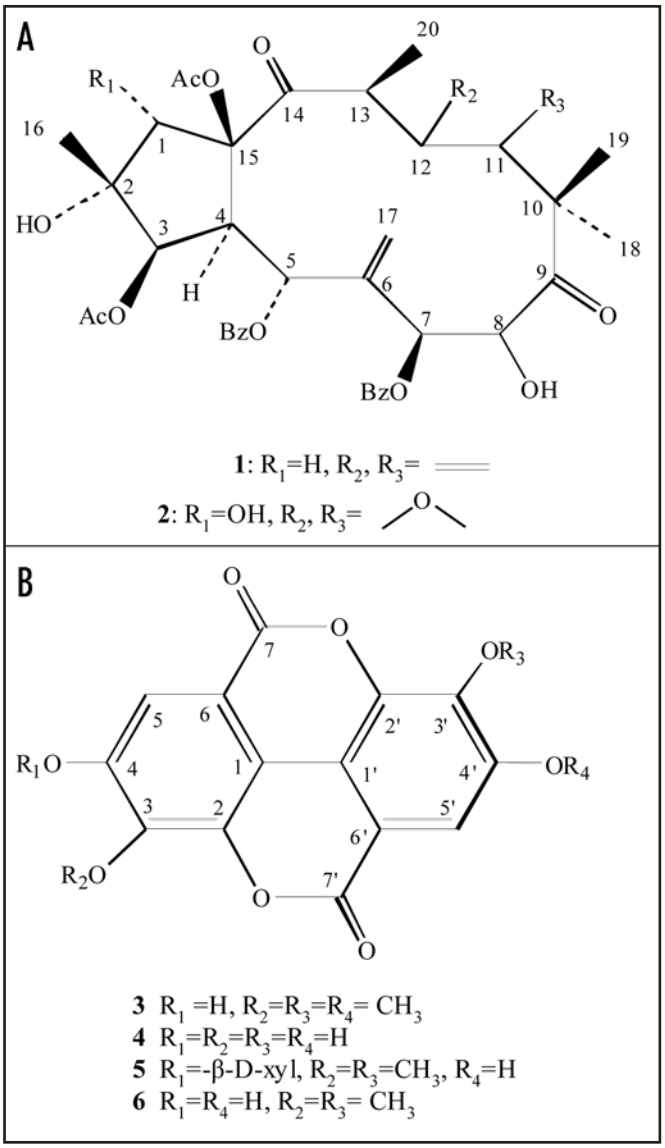

Figure 3. (A) Structures of jatrophane diterpenes identified in the chloroform extract of leafy spurge (*Euphorbia esula* L.) root material. Compound 1 = esulone A and compound 2 = kansuinine B. Kansuinine B (2) was also present in the chloroform extract of leafy spurge root exudates. (B) Structures of ellagic acid and its derivatives identified in the chloroform extract of leafy spurge (*Euphorbia esula* L.) root material. Compound 3 = 3,3',4-tri-O-methylellagic acid, 4 = ellagic acid, 5 = 4-O-[b-D-xylopyranosyl]-3,3'-di-O-methylellagic acid, and 6 = 3,3'di-O-methylellagic acid. 3,3',4-tri-O-methylellagic acid (3) was also present in the chloroform extract of leafy spurge root exudates. The xylopyranosyl ellagic acid derivative (5) and 3,3'di-O-methylellagic acid (6) were also present in the ethyl acetate extract of leafy spurge root exudates.

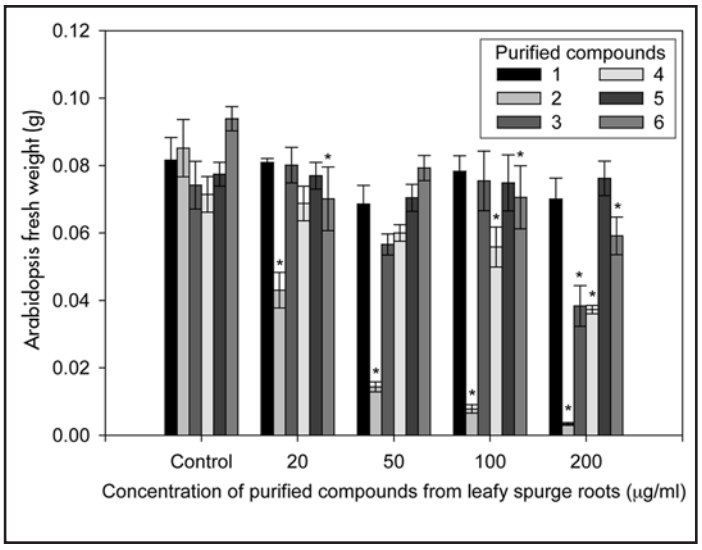

Figure 4. Phytotoxic activity of the purified compounds from leafy spurge (*Euphorbia esula* L.) on seven-day-old *Arabidopsis thaliana*. Five concentrations (0 to 200 μg ml-1) of each purified compound were applied to *A. thaliana* plants growing in liquid MS medium. Compound 1 = esulone A, 2 = kansuinine B, 3 = 3,3',4-tri-O-methylellagic acid, 4 = ellagic acid, 5 = 4-O-[b-D-xylopyranosyl]-3,3'-di-O-methylellagic acid, and 6 = 3,3'di-O-methylellagic acid. All treatments received 10 ml DMSO as a solvent. All six compounds significantly reduced *A. thaliana* biomass (1: $F_{1,18} = 1.96$, $p = 0.18$. 2: $F_{1,18} = 21.92$, $p = 0.0002$. 3: $F_{1,18} = 12.79$, $p = 0.002$. 4: $F_{1,18} = 43.02$, $p < 0.0001$. 5: $F_{1,18} = 0.00$, $p = 0.97$. 6: $F_{1,18} = 8.49$, $p = 0.009$). Means significantly lower than the DMSO controls are indicated with (*) (Dunnett's one-sided t-test, $p < 0.05$). Error bars are one standard error of the mean. N = 4.

and reducing growth at concentrations as low as 5 μg ml^{-1}. Ellagic acid (4), and to some degree its methylated derivatives (3 and 6), inhibited fresh weight, root elongation and root branching at relatively high concentrations (100 to 200 μg ml^{-1}), but did not kill seedlings at the concentrations tested.

Esulone A (1) has been identified previously in leafy spurge aboveground tissue.[8] In previous work, esulone A at 250 μg ml^{-1} caused a 29% reduction in root length in a lettuce bioassay,[8] but we found it to be inactive at 200 μg ml^{-1} in our quite different bioassay. Kansuinine B (2) appears to be much more phytotoxic than esulone A. The phytotoxicity of kansuinine B might be caused by the reactive electrophilic epoxide site at C11-C12 or by different conformations of the 12-membered macrocyclic ring, which is flexible and known to be affected by ring substitution.[18]

Different accessions of leafy spurge have been reported to contain different jatrophane diterpenes (both quantitatively and qualitatively).[9] In particular, collections from North Dakota contained quite different diterpenes than those from Oregon, Montana and Austria.[9] The chromatographic profile of the diterpenes from the Austrian plants was also quite different from those of the plants from Oregon and Montana (as well as North Dakota), which could be important in explaining why leafy spurge is not invasive in Austria. However, kansuinine B (2) was not detected in leafy spurge roots from any of the above accessions. Thus, the presence of this compound in roots of leafy spurge accessions from the native and introduced ranges needs to be reassessed.

Two ellagic acid derivatives different from those we found here (3,3'-di-O-methylellagic acid 4-β-D-xyloside and 3,3'-di-O-methylellagic acid 4-D-glucoside) have also been reported from leafy spurge, although their phytotoxicity was not examined due to poor solubility.[7] Two slightly different methylated ellagic acids (3,3',4-tri-*O*-methyl and 3,4,4'-tri-*O*-methyl) from those we found have been reported to be inhibitors of auxin transport in chestnut root cuttings, perhaps accounting for the difficulty of obtaining chestnut plants from root cuttings.[19] The decreased root branching we observed when Arabidopsis was treated with ellagic acid (and derivatives) might arise from a similar mechanism. It is also interesting to note that ellagic acid has been reported to occur in roots of *Tamarix nilotica,*[17] a congener of tamarisk (*Tamarix ramosissima* Ledeb.), an invasive species of riparian areas in the southwest United States.

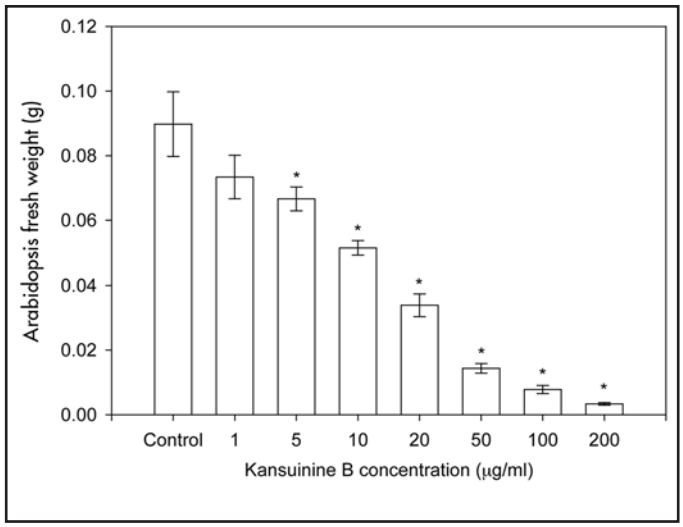

Figure 5. Phytotoxic activity of kansuinine B (2) on seven-day-old *Arabidopsis thaliana* (regression, $F_{1,30} = 42.03$, $p < 0.0001$). Eight concentrations (0 to 200 μg ml-1) of purified kansuinine B were applied to *A. thaliana* plants growing in liquid MS medium. All treatments received 10 ml DMSO as a solvent. Means significantly lower than the DMSO control are indicated with (*) (Dunnett's one-sided t-test, $p < 0.05$). Error bars are one standard error of the mean. N = 4.

The present study is unlikely to have identified all phytotoxins in leafy spurge roots. We did not purify compounds from the aqueous phase of extracted leafy spurge roots, although the aqueous phase strongly inhibited Arabidopsis seedlings (Fig. 2). Further, we purified compounds from only one of seven phytotoxic fractions from the chloroform extract of leafy spurge roots. Finally, leafy spurge roots may produce volatile phytotoxins, which our methods would not have allowed us to detect. Thus, other compounds in leafy spurge roots may be as or more phytotoxic than those we have identified.

Several of the compounds from leafy spurge roots that we have described in this paper were also present in leafy spurge root exudates, suggesting that these compounds could accumulate in the leafy spurge rhizosphere as well as leaching into the soil from decaying roots. Moreover, leafy spurge root exudates exhibited phytotoxic activity, suggesting that leafy spurge roots may exude sufficient quantities of phytotoxic compounds to influence other plants. Further studies are needed to determine whether any of the compounds that we have described here are present in the leafy spurge rhizosphere or bulk soil. If these compounds, particularly kansuinine B (2), accumulate in leafy spurge soil, they could contribute to leafy spurge invasiveness in North America. Thus, research is also needed to examine the effects of these compounds on native North American plants displaced by leafy spurge at concentrations produced by leafy spurge under natural conditions. Of particular interest is the observation that kansuinine B (2) and trimethylellagic acid (3) were both present in the chloroform-extracted leafy spurge root exudates, since these compounds seem to have complementary activities. Under natural circumstances these two types of compounds might act in coordination in the rhizosphere resulting in an overall phytotoxic effect on neighboring plants. However, further ecological studies are needed to justify this claim.

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